## REMARKS

Claims 1, 3-5, 7, 9-13 and 19-21 are all the claims pending in the present application. Claims 1, 3-5, 7, 9-13 and 19-21 have been rejected. Claims 2, 6, 8, 14-18, 22-25 have been canceled. Claims 1, 7 and 19 are amended herewith and new claim 26 has been added.

### *Claim Rejections under 35 U.S.C. §112, first paragraph*

Claims 1, 3-5, 7, 9-13 and 19-21 have been rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirements for the following reasons as set forth on pages 2-3 of the Office Action:

- the claims are drawn to a genus of bacterial autoinducer inactivation protein-encoding nucleic acids that hybridize to any nucleic acid that encodes SEQ ID NO: 2 under hybridization conditions;

- the specification does not describe any structural characteristics of the claimed nucleic acids;

- the structural features of the claimed nucleic acids are not described; and

- the only species described in the specification is SEQ ID NO: 1, which encodes SEQ ID NO 2.

In response to the rejection, Applicants submit they have satisfied the written description requirement and have reasonably conveyed to one or ordinary skill in the art that Applicants had possession of the claimed invention. Applicants have demonstrated using Southern Blot analysis that the claimed nucleic acid is conserved in many Bacillus strains. These conserved molecules exhibit similarity between 90-94% and have been cloned and shown to encode inactivation of bacterial autoinducers. (Dong et al., 2002. Appl. Environ. Microbiol. 68, 1754-1759) (a copy is attached for the Examiner's convenience). However, in an effort to advance prosecution and clarify the language of the claims, claims 1, 7 and 19 have been amended. Accordingly, it is submitted that the specification provides an adequate written description of the claimed invention. Applicants further submit that claims 3, 5, 9, 11 and 19-21 dependent from independent claims 1 and 7 comply with the written description requirement. Therefore, withdrawal of this rejection is respectfully requested.

Claims 1, 3-5, 7, 9-13 and 19-21 are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for nucleic acids encoding SEQ ID NO:2, does not reasonably provide (according to the Examiner) enablement for nucleic acids that hybridize to SEQ ID NO: 1 or that hybridize to any nucleic acid that encodes SEQ ID NO:2, vectors comprising them, cells transformed with the vector and a method of using the nucleic acids to increase disease resistance in a plant. The specification, according to the Examiner does not teach from which organisms the nucleic acid of claim 1, part c) can be isolated, or which organisms can be used as donor organisms in the method of claims 19-21, and also argued that bacterial isolate 240B1 cannot be used to isolate the nucleic acid of SEQ ID NO:1 because it is not deposited or publicly available. The Examiner also reiterates the argument that the specification does not describe the isolation of a nucleic acid that hybridizes to SEQ ID NO:1 or that hybridizes to any nucleic acid that encodes SEQ ID NO:2 from a publicly available donor organism, and undue trial and error experimentation would be required to screen all the donor organisms encompassed by the claims to identify those that have a nucleic acid that hybridizes to SEQ ID NO:1 or that hybridizes to any nucleic acid that encodes SEQ ID NO:2.

In response to the rejection, Applicants submit that they have satisfied the enablement requirement. The Examiner's analysis regarding Molina as set forth at page 8, lines 7-11 and at page 10, lines 8-12 is misguided. Molina teaches the results of two bacterial autoinducer enzymes rather than the bacterial autoinducer inactivation enzyme like AiiA. Furthermore, Applicants have demonstrated that the nucleic acid molecule in claim 1 confers strong disease resistance in transgenic plants. (Dong et al., 2001. Nature 411, 813-817) (a copy is attached for the Examiner's convenience). However, in an effort to advance prosecution, claims 1, 7 and 19 have been amended. Accordingly, it is submitted that the specification provides enablement and withdrawal of the rejection is respectfully requested.

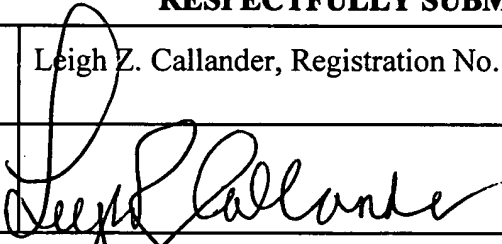### *Claim Rejections under 35 U.S.C. §112, second paragraph*

Claims 1, 3-5 and 19-21 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that

Applicant regards as the invention. The rejection is repeated for reasons of record as set forth in the Office Action mailed May 25, 2005. The Examiner argues that any nucleic acid has at least 6 potential reading frames, and thus at least 6 coding portions. In an effort to advance prosecution, claim 1 has been amended so that "the coding portion of" is replaced with "the bases that encode SEQ ID NO: 2."

For the above reasons, Applicants respectfully submit that the claims distinctly claim the subject matter of the present invention and request that the rejection under 35 U.S.C. §112, second paragraph be withdrawn.

In view of the above amendments and remarks, it is submitted that the claims are in condition for allowance. The Examiner is invited to telephone the undersigned to expedite allowance of this application.

The Commissioner is hereby authorized to charge the fee of $120.00 or credit any overpayments to Deposit Account Number 02-2135.

| RESPECTFULLY SUBMITTED, | | | | | |
|---|---|---|---|---|---|
| *Name and Reg. Number* | Leigh Z. Callander, Registration No. 55,035 | | | | |
| *Signature* | | | DATE | June 16, 2006 | |
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2977-127.AMD3.wpd

# Identification of Quorum-Quenching N-Acyl Homoserine Lactonases from *Bacillus* Species

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A range of gram-negative bacterial species use N-acyl homoserine lactone (AHL) molecules as quorum-sensing signals to regulate different biological functions, including production of virulence factors. AHL is also known as an autoinducer. An autoinducer inactivation gene, *aiiA*, coding for an AHL lactonase, was cloned from a bacterial isolate, *Bacillus* sp. strain 240B1. Here we report identification of more than 20 bacterial isolates capable of enzymatic inactivation of AHLs from different sources. Eight isolates showing strong AHL-inactivating enzyme activity were selected for a preliminary taxonomic analysis. Morphological phenotypes and 16S ribosomal DNA sequence analysis indicated that these isolates probably belong to the species *Bacillus thuringiensis*. Enzymatic analysis with known *Bacillus* strains confirmed that all of the strains of *B. thuringiensis* and the closely related species *B. cereus* and *B. mycoides* tested produced AHL-inactivating enzymes but *B. fusiformis* and *B. sphaericus* strains did not. Nine genes coding for AHL inactivation were cloned either by functional cloning or by a PCR procedure from selected bacterial isolates and strains. Sequence comparison of the gene products and motif analysis showed that the gene products belong to the same family of AHL lactonases.

N-Acyl-homoserine lactones (AHLs), also known as autoinducers, are widely conserved signal molecules that are present in the quorum-sensing systems of many gram-negative bacteria. The bacteria release, detect, and respond to accumulation of these signal molecules for synchronizing expression of particular sets of genes and for coordinating cellular activities. It has been found that AHLs are involved in regulation of a range of biological functions, including bioluminescence in *Vibrio* species (4, 13), Ti plasmid conjugal transfer in *Agrobacterium tumefaciens* (32), induction of virulence genes in *Burkholderia cepacia*, *Erwinia carotovora*, *Erwinia chrysanthemi*, *Erwinia stewartii*, *Pseudomonas aeruginosa*, and *Xenorhabdus nematophilus* (3, 6, 12, 17, 20–23, 25), regulation of antibiotic production in *Pseudomonas aureofaciens* and *E. carotovora* (6, 25), swarming motility in *Serratia liquifaciens* (14), and biofilm formation in *Pseudomonas fluorescens* and *P. aeruginosa* (1, 8). More bacterial species are known to produce AHLs, but the relevant biological functions have not been investigated (2, 5, 11).

AHL quorum-sensing signals are a fascinating group of molecular targets for genetic and chemical manipulation. These molecules are highly conserved; they have the same homoserine lactone moiety but differ in the length and structure of the acyl side chain. Although different target genes are regulated by AHLs, the basic mechanisms of AHL biosynthesis and gene regulation seem to be conserved in different bacterial species. The general feature of AHL-mediated gene regulation is cell population-dependent regulation, which is known as quorum sensing. The concentration of an AHL increases along with the growth of bacterial cells. When the AHL concentration reaches a threshold level, it triggers target gene expression

(16). The biological functions regulated by AHLs are of considerable scientific and economic importance. New approaches for up or down regulation of bacterial quorum-sensing systems would be of significant interest not only for scientific purposes but also for practical applications.

We recently reported cloning of a novel gene, $aiiA_{240B1}$ coding for an AHL-inactivating enzyme ($AiiA_{240B1}$), from the gram-positive bacterium *Bacillus* sp. strain 240B1 (9). $AiiA_{240B1}$ inactivates an AHL by hydrolyzing its lactone bond and was designated AHL lactonase (10). Expression of $aiiA_{240B1}$ in transformed *E. carotovora* strain SCG1, a pathogen that causes soft rot disease in many plants, significantly reduces release of AHL, decreases extracellular pectrolytic enzyme activities, and attenuates pathogenicity for potato, eggplant, Chinese cabbage, carrot, celery, cauliflower, and tobacco (9). Transgenic plants expressing AHL lactonase exhibit significantly enhanced resistance to *E. carotovora* infection and delayed development of soft rot symptoms (10). AHL-inactivating mechanisms appear to be widely distributed. An isolate of *Variovorax paradoxus* has been reported to use AHL molecules as energy and nitrogen sources, indicating that AHL-degrading enzymes are present in this organism (19). In this study, we focused on the biodiversity of AHL-inactivating enzymes and bacterial strains. We identified more than 20 bacterial isolates and strains capable of AHL inactivation obtained from soil and plant samples and from a laboratory bacterial culture collection. Nine genes coding for AHL inactivation (*aiiA*) were cloned from gram-positive *Bacillus* species and characterized. Biochemical and molecular analyses showed that the enzymes encoded by these genes belong to the same family of AHL lactonases.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are described in Table 1. Screening of bacterial isolates capable of inacti-

TABLE 1. Bacterial strains and plasmids[a]

| Bacterial strain or plasmid | Serotype or related characteristics | Source or reference |
|---|---|---|
| **Bacteria** | | |
| *Bacillus* strains | | |
| B1 (*B. thuringiensis* subsp. *thuringiensis*) | H$_1$ | BGSC 4A3 |
| B2 (*B. thuringiensis* subsp. *kurstaki*) | H$_{3a3b}$ | BGSC 4D1 |
| B12 (*B. thuringiensis* subsp. *aizawai*) | H$_7$ | BGSC 4J4 |
| B17 (*B. thuringiensis* subsp. *wuhanensis*) | No flagella | Mycogen PSS2A1 |
| B18 (*B. thuringiensis*) | | Lab collection |
| B20 (*B. thuringiensis*) | | Lab collection |
| B21 (*B. thuringiensis*) | | Lab collection |
| B22 (*B. thuringiensis* subsp. *kurstaki*) | H$_{3a3b}$, plasmidless | Lab collection |
| B23 (*B. thuringiensis* subsp. *israelensis*) | H$_{14}$, plasmidless | BGSC 4Q7 |
| B25 (*B. cereus*) | | Lab collection |
| 14579 (*B. cereus*) | | ATCC 14579 |
| 6462 (*B. mycoides*) | | ATCC 6462 |
| 269 (*B. fusiformis*) | | Lab collection |
| B29 (*B. sphaericus*) | | BGSC 12A4 |
| *Agrobacterium tumefaciens* NT1 | *traR tra::lacZ749*, indicator strain | 24 |
| *Escherichia coli* DH5α | F$^-$φ80d *lacZΔM15 endA1 hsdR17* (r$_k^-$ m$_k^-$) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*) | 26 |
| **Plasmids** | | |
| pLARF3 | Tc$^r$ | 27 |
| pBluescript SK+ | Ap$^r$ | Stratagene |
| pGEM-7Zf(+) | Ap$^r$ | Promega |
| pGEM-T | Ap$^r$ | Promega |
| pLARF3-*aiiA*$_{COT1}$ | Tc$^r$, 24-kb *Eco*RI fragment from COT1 genomic DNA in pLARF3 | This study |
| pGEM-*aiiA*$_{COT1}$ | Ap$^r$, 5-kb *Eco*RI fragment from pLARF3-*aiiA*$_{COT1}$ in pGEM-7Zf(+) | This study |
| pBS-*aiiA*$_{COT1}$ | Ap$^r$, 1.3-kb *Bam*HI fragment from pGEM-*aiiA*$_{COT1}$ in pBluescript SK | This study |

[a] BGSC, *Bacillus* Genetic Stock Centre; ATCC, American Type Culture Collection; Lab collection, laboratory collection strain; Tc$^r$, tetracycline resistance; Ap$^r$, ampicillin resistance. Eight new bacterial isolates are described in the text.

vating AHL has been described previously (9). All isolates and *Bacillus* strains were grown at 28°C in Luria-Bertani medium. *Escherichia coli* strains were grown at 37°C. Ampicillin (100 μg/ml) and tetracycline (15 μg/ml) were added to the medium when they were required. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Promega) was included in the medium at a concentration of 50 μg/ml for detection of β-galactosidase activity.

AHL bioassay. To determine AHL-inactivating activity, N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) was added at a final concentration of 20 μM to an overnight bacterial culture which was diluted to an optical density at 600 nm (OD$_{600}$) of 1.1. The reaction mixture was incubated at 28°C for different times as indicated below. The amount of OHHL remaining in the supernatant or reaction mixture was then determined as previously described (9, 31). *A. tumefaciens* strain NT1 containing a *lacZ* fusion in the *tra* gene of pTiC58 was used as an indicator strain for AHL activity (24). AHL-inactivating activity was expressed as the number of picomoles of OHHL inactivated per hour per unit of OD$_{600}$ of cell culture.

Cloning of *aiiA*$_{COT1}$ gene. Genomic DNA was purified from bacterial isolate COT1 and digested partially with *Eco*RI. DNA fragments were ligated to the dephosphorylated *Eco*RI site of cosmid vector pLAFR3 (27). Ligated DNA was packaged and transfected into *E. coli* DH5α. Cosmid clones with AHL-inactivating activity were identified by using the bioassay method described above. Subcloning into sequencing vector pGEM-7Zf(+) or pBluescript SK was carried out by routine techniques (26). Sequencing was performed for both strands by using an ABI Prism dRhodamine terminator cycle sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems).

PCR procedure for cloning the *aiiA* gene from other *Bacillus* species. Genomic DNA samples were used as PCR templates. The primers were designed based on the identical sequences of the 5′ ends of the *aiiA*$_{240B1}$ and *aiiA*$_{COT1}$ open reading frames (ORFs) and the conserved region 119 nucleotides after the stop codon. The forward primer was 5′-ATG GGA TCC ATG ACA GTA AAG AAG CTT TAT-3′, and the reverse primer was 5′-GTC GAA TTC CTC AAC AAG ATA CTC CTA ATG-3′. Each PCR was performed for 30 cycles consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. PCR products were purified by using a QIAquick PCR purification kit (QIAGEN), and the purified PCR fragments were ligated to the pGEM-T vector (Promega). The clones which conferred AHL-inactivating activity were used for further study. At least two clones of each gene were sequenced to avoid misreading due to PCR error. A database search was performed by using the BLASTA search algorithm. The DNASTAR sequence analysis software package (DNASTAR Inc.) and the GCG sequence

analysis software (Genetics Computer Group, Madison, Wis.) were used for sequence analysis at the nucleotide and peptide levels.

Southern blot analysis. Three bacterial isolates and 15 *Bacillus* strains, including 12 *B. thuringiensis* strains, 2 *B. cereus* strains, and 1 *B. sphaericus* strain, were used for Southern blot analysis. Genomic DNA (20 μg) that was digested with *Eco*RI was separated by electrophoresis in a 0.8% agarose gel and transferred onto a Hybond-N$^+$ membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The 1.4-kb *Bam*HI fragment containing the *aiiA*$_{COT1}$ coding region was labeled with digoxigenin-11-dUTP and used as a probe for hybridization. After hybridization at 65°C, the membrane was washed twice in 2× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and this was followed by two washes in 0.1× SSC–0.1% SDS at 65°C for 15 min. A nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution was used as the color substrate as recommended by the manufacturer's protocol (Boehringer Mannheim).

Protein extraction and Western blot analysis. Bacterial cells were grown overnight at 28°C (for *Bacillus* strains) or 37°C (for *E. coli*) in Luria-Bertani medium. Cells were harvested by centrifugation at 4°C. Each cell pellet was washed once with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$; pH 7.4) and then resuspended in ice-cold phosphate-buffered saline and sonicated on ice. Cell debris was removed by centrifugation. The protein concentration was determined by the Bradford method with reagents obtained from Bio-Rad (Hercules, Calif.). Proteins (25 μg) were separated by SDS-12% polyacrylamide gel electrophoresis, transferred onto a polyvinyidene difluoride membrane (Bio-Rad), and then probed with polyclonal anti-(AHL lactonase) antibodies (10), followed by alkaline phosphatase-conjugated secondary antibodies. Purified AHL lactonase (10 ng) encoded by *aiiA*$_{240B1}$ was used as a control in the immunoblot analysis.

Nucleotide sequence accession numbers. The nucleotide sequences of *aiiA*$_{COT1}$, *aiiA*$_{B1}$, *aiiA*$_{B2}$, *aiiA*$_{B17}$, *aiiA*$_{B18}$, *aiiA*$_{B20}$, *aiiA*$_{B21}$, *aiiA*$_{B22}$, and *aiiA*$_{B25}$ have been deposited in the GenBank database under accession numbers AF350927 to AF350935.

## RESULTS

**Isolation of bacteria capable of inactivating AHL.** We screened more than 800 bacterial isolates obtained from soil
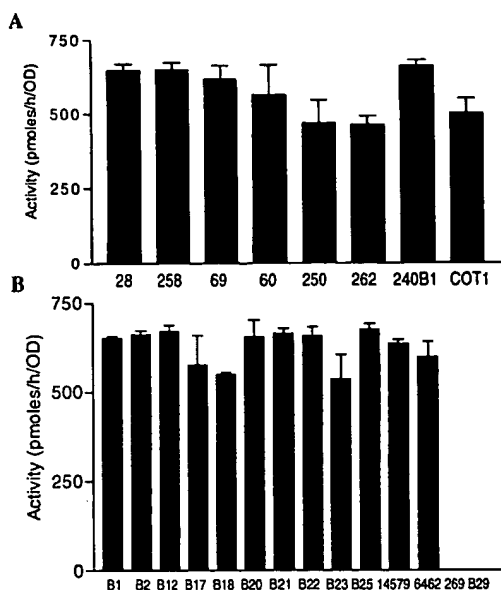
FIG. 1. AHL inactivation analysis of bacterial isolates and strains.
(A) AHL-inactivating activities of newly identified bacterial isolates;
(B) AHL-inactivating activities of previously described *Bacillus* strains
(as shown in Table 1). A cell suspension culture ($OD_{600}$, 1.1) prepared
from each bacterial isolate or strain was mixed with an equal volume
of 40 μM OHHL and then incubated at 28°C for 30 min. The amount
of OHHL remaining in the supernatant was determined as previously
described (9, 31). The activities are expressed in picomoles of OHHL
inactivated per hour per unit of $OD_{600}$ of bacterial culture. The values
are means ± standard deviations based on four replicates.

and plants for AHL-inactivating activity. By incubating fresh
bacterial cultures with OHHL, we identified more than 20
bacterial isolates that exhibited AHL-inactivating activity dur-
ing the preliminary screening. Eight of these isolates showing
strong activity were selected and characterized at the biochem-
ical and molecular levels. The AHL-inactivating activities were
variable, ranging from 480 to 680 pmol/h/unit of $OD_{600}$ (Fig.
1A). To characterize these isolates, their 16S ribosomal DNA
(rDNA) sequences were analyzed by PCR amplification and
subsequent sequencing. The results showed that the 16S rDNA
sequences of these isolates are highly homologous to the 16S
rDNA sequence of *B. thuringiensis* (data not shown). To con-
firm the 16S rDNA sequence similarity, we sequenced the 16S
rDNA from a known *B. thuringiensis* strain, *B. thuringiensis*
subsp. *thuringiensis* BGSC 4A3. A sequence comparison re-
vealed that the levels of identity between the BGSC 4A3 rDNA
and the rDNA of these isolates were 97.8 to 98.1%. The other
characteristics exhibited by these isolates (gram positive, peri-
trichous, straight rods, endospore formation) also indicated
that they are the members of *B. cereus* group in the genus
*Bacillus*.

All of the *B. thuringiensis* and closely related strains tested
had AHL-inactivating activity. The *B. cereus* group includes
*B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis* (29).
We selected 14 known *Bacillus* strains, including 9 *B. thur-
ingiensis* strains, 2 *B. cereus* strains, 1 *B. mycoides* strain, 1
*B. fusiformis* strain, and 1 *B. sphaericus* strain, to test whether
these species have the ability to inactivate AHL. All of the
bacterial strains tested except the *B. fusiformis* and *B. sphaeri-*

*cus* strains eliminated AHL with levels of enzyme activity
(range, 534 to 674 pmol/h/unit of $OD_{600}$) (Fig. 1B) similar to
those of the newly identified AHL-inactivating bacterial iso-
lates (Fig. 1A).

It is known that many *B. thuringiensis* strains contain plas-
mids. Our data suggest that AHL-inactivating genes are locat-
ed in chromosomal DNA and not in plasmids, because *B. thur-
ingiensis* subsp. *kurstaki* strain B2 (= BGSC 4D1) and its
plasmidless derivative, strain B22, exhibited similar levels of ac-
tivity. A second plasmidless strain, strain B23 (= BGSC 4Q7)
(http://bacillus.biosci.ohio-state.edu/), belonging to *B. thurin-
giensis* subsp. *israelensis*, was also capable of inactivating AHL
(Fig. 1B).

**Functional cloning of *aiiA*$_{COT1}$ gene.** Bacterial cells of strain
COT1 eliminated OHHL (20 μM) completely after 2 h of
incubation at 28°C, but bacterial cells killed by boiling for
5 min failed to inactivate OHHL (Fig. 2), indicating that there
is an enzymatic inactivation mechanism. To identify the gene
coding for AHL inactivation in COT1, a cosmid library was
constructed and used for functional screening. One clone
(pLARF3-*aiiA*$_{COT1}$) showing AHL-inactivating activity in the
bioassay was identified after we screened about 1,000 cosmid
clones. Restriction analysis showed that this clone contained
a 24-kb insert. The five fragments generated by complete di-
gestion with *Eco*RI were subcloned into the pGEM-7 vector.
A bioassay with the subclones showed that one subclone,
pGEM-*aiiA*$_{COT1}$ with an 5-kb insert, conferred AHL-inactivat-
ing activity. Further subcloning led to identification of clone
pBS-*aiiA*$_{COT1}$ containing a 1.3-kb *Bam*HI fragment which
codes for AHL inactivation. Complete sequence analysis of
clone pBS-*aiiA*$_{COT1}$ showed that there was a 750-bp ORF
which encoded a 250-amino-acid protein. Expression of this
ORF in *E. coli* confirmed that it encoded a functional AHL-
inactivating enzyme, designated AiiA$_{COT1}$. At the peptide se-
quence level, AiiA$_{COT1}$ exhibited 91% identity to AiiA$_{240B1}$
(9), which has recently been identified as a novel AHL lacto-
nase (10).

**Southern blot detection of *aiiA*$_{COT1}$ homologues in *Bacillus*
species.** As all of the bacterial isolates and strains capable of
inactivating AHL that were tested belong to *B. thuringiensis* or
closely related taxa and the *aiiA*$_{240B1}$ and *aiiA*$_{COT1}$ genes
showed a high level of similarity, it is very likely that the *aiiA*
gene is highly conserved in *B. thuringiensis* and closely related
species. DNA hybridization analysis was performed using the
*aiiA*$_{COT1}$ gene as a probe. Genomic DNA were isolated from



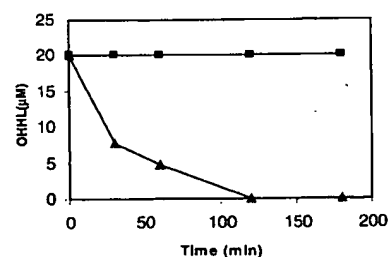FIG. 2. Enzymatic inactivation of OHHL by suspension culture of
COT1. Equal volumes of a cell suspension culture ($OD_{600}$, 1.1) and 40
μM OHHL were mixed and incubated at 28°C (▲). A boiled culture
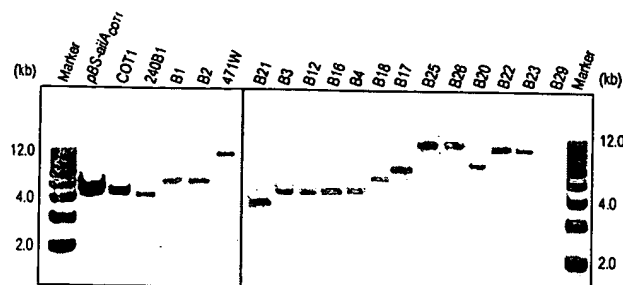mixed with 40 μM OHHL was used as a control (■).

FIG. 3. DNA hybridization analysis of different bacterial isolates and known *Bacillus* species. Total DNA was digested with *Eco*RI, fractionated in a 0.8% agarose gel, blotted, and then probed with a digoxigenin-labeled 1.3-kb fragment containing the *aiiA*<sub>COT1</sub> gene. All of the strains used except B26 (*B. cereus*) and B29 (*B. sphaericus*) are *B. thuringiensis* strains.

18 selected bacterial strains and isolates, including 13 *B. thuringiensis* strains, 1 *B. cereus* strain, 1 *B. sphaericus* strain, and 3 isolates. The results (Fig. 3) showed that one hybridizing band was clearly produced by all of the strains tested except *B. sphaericus* B29, which was unable to inactivate AHL (Fig. 1B). These results indicate that there is an *aiiA*<sub>COT1</sub> homologue in all of the strains of *B. thuringiensis* and the closely related species *B. cereus* tested. This is consistent with the bioassay data (Fig. 1). To detect AHL lactonase in these AHL-inactivating bacterial strains, the soluble proteins extracted from the strains were subjected to Western blot analysis using a rabbit anti-AHL lactonase antiserum. As shown in Fig. 4A, immunoblot signals were detected in the strains.

**Cloning of *aiiA* genes from other selected *Bacillus* strains.** As the genes for AHL inactivation in *Bacillus* strains 240B1 and COT1 are highly conserved, a PCR approach was used to clone the AHL lactonase genes from selected *B. thuringiensis* and *B. cereus* strains. Genomic DNA isolated from *B. thuringiensis* strains B1, B2, B17, B18, B20, B21, and B22 and *B. cereus* strain B25 were used as templates. Purified PCR fragments were cloned into the pGEM-T vector (Promega). The resulting eight clones, which showed AHL-inactivating activity in the bioassay, were used to obtain the sequences of both strands. The sequences of the eight AHL-inactivating genes cloned from strains B1, B2, B17, B18, B20, B21, B22, and B25 all contained a 750-bp ORF which encoded a 250-amino-acid



FIG. 5. Phylogenetic tree analysis and levels of amino acid identity of AHL lactonases. The phylogenetic tree was produced with the DNASTAR sequence analysis software (DNASTAR Inc.). Distances are shown below the tree. The levels of amino acid identity of AHL lactonases from strains COT1, B1, B2, B17, B18, B20, B21, B22, and B25 to the AHL lactonase from strain 240B1 are shown on the right.

protein. Western blot analysis confirmed that these genes were expressed in *E. coli* (Fig. 4B).

**Motif analysis of AHL-inactivating genes.** Sequence analysis indicated that 10 *aiiA* genes cloned from *Bacillus* species were highly conserved, with amino acid identities ranging from 90.4 to 94.0% compared with the amino acids of AHL lactonase (Fig. 5). As determined by a BLAST CD search of Conserved Domain Databases (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), two conserved regions were found by aligning AHL lactonases with metallo-beta-lactamase (pfam00753, Conserved Domain Databases) and zinc-containing glyoxalase II (Y08357, GenBank) (Fig. 6A). Several histidine and glutamate residues in these two conserved regions are known to be essential for zinc binding and enzyme activity of metallohydrolases (7, 30). Site-directed mutagenesis has shown previously that residues H106, D108, H109, and H169 of AiiA<sub>240B1</sub> are necessary for AHL lactonase activity (9). Based on sequence alignment, we performed site-directed mutagenesis with several other conserved amino acid residues, including D191, H235, and D236. We replaced these residues with serine residues. The results showed that the D191S mutation resulted in a complete loss of enzyme activity, whereas the H235S and D236S mutations did not affect the enzyme activity (Fig. 6B). These data established a motif for AHL lactonase, $^{106}$HXDH-59 amino acids-H$^{169}$-21 amino acids-D$^{191}$, which is essential for enzyme activity.
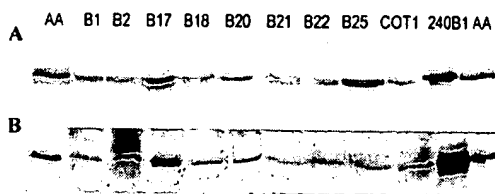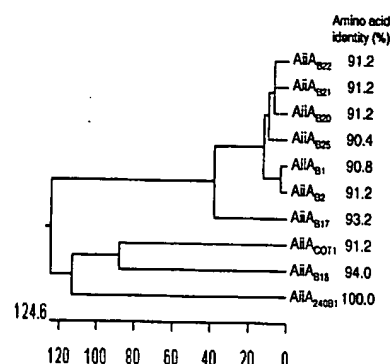


FIG. 4. Western blot analysis. (A) Western blot analysis of the total proteins extracted from bacterial isolates and strains capable of inactivating AHL. Soluble proteins from 10 bacterial isolates and strains and 10 ng of purified AHL lactonase (lanes AA) were separated by SDS-polyacrylamide gel electrophoresis and were visualized by anti-AHL lactonase antibody coupling in a standard alkaline phosphatase immunoassay. (B) Western blot analysis of the total proteins extracted from *E. coli* strains containing the *aiiA* gene isolated from different strains.

## DISCUSSION

Nine genes encoding AHL-inactivating enzymes were cloned from gram-positive bacterial isolates and strains. These genes exhibited high levels of homology to *aiiA*<sub>240B1</sub>, which encodes an AHL lactonase (9, 10). Similar to the AHL lactonase encoded by *aiiA*<sub>240B1</sub>, the putative zinc-binding motif that has catalytic importance is conserved in these newly identified AHL-inactivating enzymes (Fig. 6A). Data suggest that these enzymes are members of the AHL lactonase family. Detailed characterization of the relationship between the structure and activity of these enzymes would be useful for elucidation of the enzymatic mechanism.

**A**

```
Lac      ILTRARADHIGGVPELL-51aa-TPGHTPGSIVVYLPEEK---VLFTGDLLFS
Gly      LNTEHHDDHIGGNAELK-41aa-TPGHTQGHISFYFPGSATIFTGDLIYSLSC
AiiA_B1  ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLPIETEQSGSVLLMIDASYT
AiiA_B2  ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLPIETEQSGSVLLTIDASYT
AiiA_B20 ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLPIETEQSGSVLLTIDASYT
AiiA_B22 ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLPIETEQSGSVLLTIDASYT
AiiA_B21 ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLPIETDNSGSVLLTIDASYT
AiiA_B25 ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLPIETEQSGSVLLTIDASYT
AiiA_B17 ISSHLHFDHAGGNGAFT-48aa-TPGHSPGHQSLLIETEKSGLVLLTIDASYT
AiiA_B18 ISSHLHFDHAGGNGAFL-48aa-TPGHSPGHQSLLIETEKSGPVLLTIDASYT
AiiA_COT ISSHLHFDHAGGNGAFI-48aa-TPGHSPGHQSLLIETEKSGVVLLTIDASYT
AiiA_2402 ISSHLHFDHAGGNGAFI-48aa-TPGHTPGHQSLLIETEKSGPVLLTIDASYT
          • ••              •                          •
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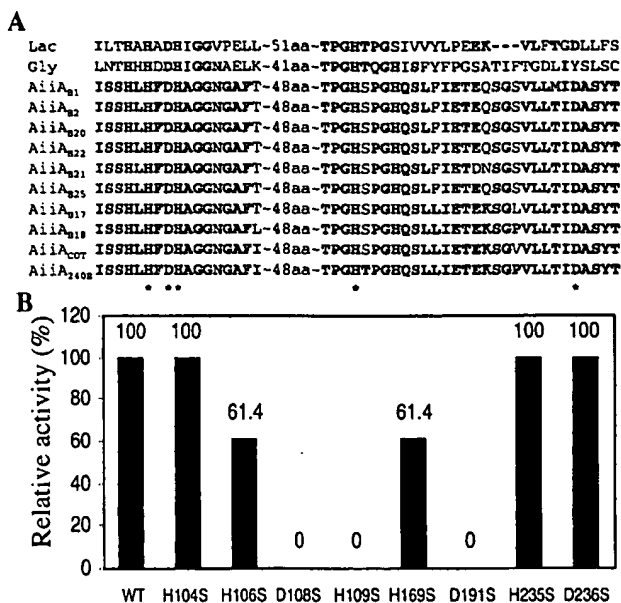
**B**



FIG. 6. Alignment of sequences of the conserved regions and site-directed mutagenesis of AHL lactonase. (A) Alignment of the conserved regions of *Bacillus* AHL lactonases and portions of two other proteins, the metallo-beta-lactamase (Conserved Domain Databases accession no. pfam00753) amino acid sequence from residue 49 to residue 134 (Lac) and the *Arabidopsis* glyoxalase II (GenBank accession no. U90928) amino acid sequence from residue 128 to residue 214 (Gly). The regions of the *Bacillus* AHL lactonases examined included amino acid residues 101 to 195. The amino acids identical to amino acids in AiiA$_{2402}$B are indicated by boldface type. The amino acid residues necessary for AHL lactonase activity are indicated by asterisks. aa, amino acids. (B) Site-directed mutagenesis of AHL lactonase. H, histidine; D, aspartic acid; S, serine. The number in each designation indicates the position of the corresponding amino acid in the AHL lactonase peptide. Histidine and aspartic acid were replaced by serine by site-directed mutagenesis (see reference 9 for an explanation of the method). The values above the bars are the relative activities of the mutants.

Our results showed that the AHL-inactivating bacterial isolates and strains belong to *B. thuringiensis*, *B. cereus*, and *B. mycoides*, whereas two *Bacillus* species, *B. fusiformis* and *B. sphaericus*, did not exhibit detectable AHL-inactivating activity. By searching microbial genome BLAST databases, we found a homologue (gnl/TIGR_1392/banth_2063) in *B. anthracis* in which 89.1% the nucleotides are identical to those in the *aiiA*$_{COT1}$ coding sequence, suggesting that *B. anthracis* may contain a similar enzyme that inactivates AHLs. It is known that *B. anthracis*, *B. thuringiensis*, *B. cereus*, and *B. mycoides* are closely related and belong to the *B. cereus* group in the genus *Bacillus* (29). The role of AHL lactonase in these *Bacillus* species is not clear. Unlike most gram-negative bacteria that produce AHL quorum-sensing signals, some gram-positive bacteria produce γ-butyrolactones as quorum-sensing signals, which are structurally quite similar to AHLs (28). It would be interesting to investigate whether AHL lactonases also inactivate the γ-butyrolactones, how widely AHL-inactivating genes are conserved in the microbial community, and the roles of these genes in bacterial interaction and ecology.

*B. thuringiensis* has been used extensively as a microbial insecticide in last few decades (15, 18). However, most of

important insecticidal *B. thuringiensis* strains have not been exploited for disease control because they usually do not produce antibiotics that are effective against bacteria and fungi. In this study, we found that all of the *B. thuringiensis* strains tested are capable of inactivating AHL. Because short-side-chain AHLs diffuse easily into bacterial cells, *B. thuringiensis* could eliminate AHL constantly from its surroundings in order to avoid accumulation of AHLs that activate virulence genes. It is probable that insecticidal *B. thuringiensis* strains could be used as biocontrol agents against bacterial diseases which are mediated by the AHL quorum-sensing signals.

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present in the tissues[21]. This limitation results in late-stage diagnosis, in most cases from brain tissue obtained at autopsy[20,21]. However, as shown by infectivity studies, the infectious agent is present pre-symptomatically in many tissues in addition to the brain[21,22]. Thus, early detection of PrP[Sc] from non-brain sources should be possible with highly sensitive methods[21]. Our findings constitute a strategy to detect low quantities of PrP[Sc], by means of amplifying undetectable amounts of the protein to a detectable level. The PMCA procedure can be combined with any of the existing detection systems to reach a further reduction of detection threshold. Preliminary results indicate that the PMCA system can also be applied to human brain samples obtained from sporadic CJD (G.P.S. and C.S., unpublished observations). Therefore, the PMCA method opens a new possibility for TSE diagnosis that could be applied to systemic tissues or fluids during the pre-symptomatic phase of the disease. □

## Methods

### Preparation of brain homogenates

Brains from healthy rats (F.344) and Syrian golden hamsters healthy or infected with the adapted scrapie strain 263 K were obtained after decapitation and immediately frozen in dry ice and kept at −80 °C until used. Brains were homogenized in PBS buffer containing protease inhibitors (Complete cocktail from Boehringer Mannheim) at a 1× final concentration. Detergents (0.5% Triton X-100, 0.05% SDS, final concentrations) were added and samples clarified with low-speed centrifugation (1,000g) for 1 min, using an Eppendorf 5415 centrifuge.

### Cyclic amplification

Serial dilutions of the scrapie brain homogenate were made directly in the healthy brain homogenate. We incubated 60 μl of these dilutions at 37 °C with agitation. Every hour one cycle of sonication (five pulses of 1 s each) was done using a microsonicator (Bandelin Electronic, Sonopuls) with the probe immersed in the sample and the power setting fixed at 40%. These cycles were repeated 5–40 times.

### Detection of PrPres

The samples were digested with proteinase K (100 μg ml⁻¹ for 60 min at 37 °C) and the reaction was stopped with 50 mM phenyl-methyl sulphonyl fluoride. Samples were separated by SDS–PAGE and electroblotted into nitrocellulose membrane in 3-(cyclo-hexylamino)-1-propane sulphonic acid or Tris-glycine transfer buffer with 10% methanol during 45 min at 400 mA. For immunoblotting, the membranes were blocked with 5% non-fat milk and incubated for 2 h with the monoclonal antibody 3F4 (ref. 23) (1:50,000). Four washes of 5 min each were performed with PBS and 0.3% Tween20 before incubation with secondary anti-mouse antibody labelled with horseradish peroxidase (1:5,000) for 1 h. After washing, the reactivity in the membrane was developed with an ECL Chemilumicense Kit (Amersham) according to the manufacturer's instructions. Densitometric analyses of western blots were performed with the program SigmaGel v1.0 (Jandel Scientific). The concentration of PrP[Sc] was estimated by densitometric analysis and comparison with pure recombinant hamster PrP, the concentration of which was determined by amino-acid analysis.

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# Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase

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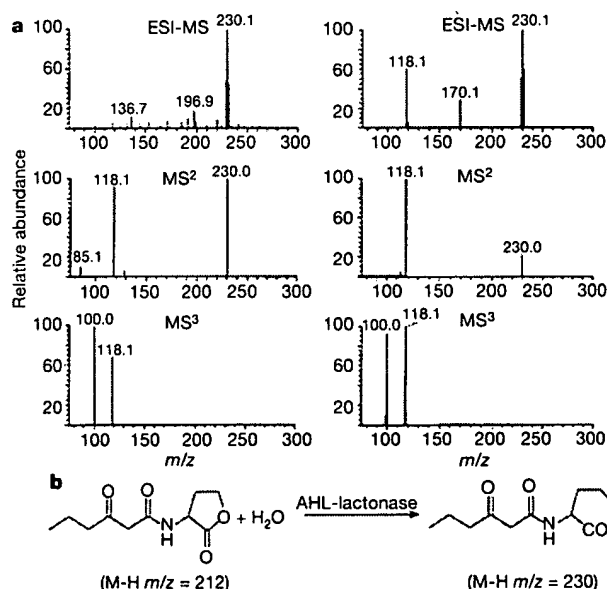* These authors contributed equally to this work

Bacterial cells sense their population density through a sophisticated cell–cell communication system and trigger expression of particular genes when the density reaches a threshold. This type of gene regulation, which controls diverse biological functions including virulence, is known as quorum sensing[1,2]. Quorum-sensing signals, such as acyl-homoserine lactones (AHLs), are the essential components of the communication system. AHLs regulate virulence gene expression in a range of plant and animal (including human) bacterial pathogens[3–9]. AHL-producing tobacco restored the pathogenicity of an AHL-negative mutant of Erwinia carotovora[10]. Different bacterial species may produce different AHLs, which vary in the length and substitution of the acyl chain but contain the same homoserine lactone moiety. Here we show that the acyl-homoserine lactonase (AHL-lactonase), a new enzyme from Bacillus sp.[11], inactivates AHL activity by hydrolysing the lactone bond of AHLs. Plants expressing AHL-lactonase quenched pathogen quorum-sensing signalling and showed significantly enhanced resistance to E. carotovora infection. Our results highlight a promising potential to use quorum-sensing signals as molecular targets for disease control, thereby broadening current approaches for prevention of bacterial infections.

Target genes regulated by AHLs are extremely varied and regulatory mechanisms are probably diversified[12–14]; however, the general mechanism of AHL-mediated quorum-sensing signalling is highly conserved. In general, each bacterial cell produces a basal level of AHLs that move in and out of cell membranes through diffusion or active transportation (ref. 15 and L.-H.Z., unpublished data). When AHLs reach a threshold concentration owing to

# letters to nature

higher bacterial population density, they interact with a LuxR family transcription factor, and initiate expression of particular genes[13,14,16]. Recently, we showed that the *aiiA* gene from a Gram-positive *Bacillus* sp. 240B1 encodes an enzyme capable of inactivating the three AHLs tested[11]; also a bacterial isolate of *Variovorax paradoxus* has been identified that degrades AHLs and releases homoserine lactone, indicating the presence of an aminoacylase[17]. These findings suggest that it could be possible to establish a generic 'quorum-quenching' approach to control bacterial infections, that is, to paralyse quorum-sensing systems of bacterial pathogens through inactivation of quorum-sensing signals.

To determine how the enzyme encoded by *aiiA* inactivates AHL signals, we first tested N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which regulates expression of virulence genes in a plant pathogen *Erwinia carotovora* pv. *carotovora*[3,5]. We digested OHHL with the enzyme and analysed the reaction products with high-performance liquid chromatography (HPLC) and mass spectrometry. Enzymatic digestion of OHHL resulted in only one product fraction, which was more hydrophilic than OHHL, as determined by HPLC analysis. Electrospray ionization mass spectrometry (ESI-MS) analysis of this product showed a strong quasimolecular (M-H) ion at an $m/z$ (mass-to-charge ratio) of 230.1 (Fig. 1a; left column, top), suggesting that the enzymatic action on OHHL (M-H ion $m/z$ of 212) leads to a mass increase of 18 in its product, corresponding to a water molecule. This is in agreement with the chemical composition of the lactone-opened OHHL, namely N-(3-oxohexanoyl)-L-homoserine (M-H ion $m/z$ of 230). Tandem mass spectrometry of the parent ion at $m/z$ of 230 (MS$^2$) showed a daughter ion of 118.1 (Fig. 1a; left column, middle), consistent with the formula of $C_4H_9NO_3$ (homoserine, M-H ion $m/z$ of 118.1). Further analysis of the ion of $m/z$ of 118.1 (MS$^3$) identified its daughter ion of $m/z$ of 100 (Fig. 1a; left column, bottom). Homoserine produced a parent ion with a $m/z$ of 118.1 and a daughter ion with a $m/z$ of 100 (data not shown). To further confirm the analysis, we produced N-(3-oxohexanoyl)-L-homoserine from its corresponding lactone and found that its mass spectrum was identical

to that of the hydrolysis product of OHHL, showing a parent ion $m/z$ of 230 in ESI-MS and daughter ions at $m/z$ of 118 and 100 in tandem MS (Fig. 1a, right column). HPLC analysis showed that the synthetic N-(3-oxohexanoyl)-L-homoserine has an identical HPLC retention time as the OHHL hydrolytic product.

We next analysed the enzyme-digested products of three other AHLs with differences in acyl-chain length and substitution at the C3 position, including N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), N-butanoyl-L-homoserine lactone (BHL), and N-(3-oxooctanoyl)-L-homoserine lactone (OOHL). OdDHL and BHL regulate production of virulence determinants in *Pseudomonas aeruginosa*[18,19], a human pathogen causing fatal infections of the lung and of burns. OOHL controls Ti plasmid conjugal transfer in *Agrobacterium tumefaciens*[20]. Results showed again that each of their molecular masses had been increased by 18 after the enzyme reaction (data not shown). In all cases, controls lacking the enzyme showed negligible delactonization. The results demonstrate that the enzyme encoded by *aiiA*, designated previously as AiiA[11], is an AHL-lactonase that hydrolyses the ester bond of the homoserine lactone ring of AHLs (Fig. 1b). The minimum concentrations for detectable biological activity of delactonized products of OHHL and OdDHL were 500 μM and 1000 μM, respectively, indicating a 1,667 times decrease in activity in comparison with OHHL and OdDHL. The residue activity of these delactonized products is unlikely to be due to spontaneous relactonization or formation of methyl ester derivatives, as HPLC and ESI-MS analysis did not detect the corresponding fractions even after incubation for two days with or without bioassay strain.

To test the effect of AHL-lactonase on bacterial infection, we introduced *aiiA* and an *sp–aiiA* fusion gene (see Methods), carried by constructs pBI-AI and pBI-SPAI, respectively (Fig. 2a), into the genome of tobacco and potato by means of *Agrobacterium*-mediated transformation[21,22]. The *sp–aiiA* fusion gene targets AHL-lactonase to the intercellular space of plant tissues where *E. carotovora* initiates infection. We randomly selected 38 kanamycin-resistant primary transgenic lines of tobacco (20 with *aiiA* and 18 with *sp–aiiA*) and 35 lines of potato (29 with *aiiA* and 6 with *sp–aiiA*) for further analysis, along with 10 controls consisting of 5 untransformed and 5 pBI-GFP (construct with green fluorescent protein coding region) transformed tobacco and potato plants.

RNA hybridization assays showed that the *aiiA* transcript was detected in all *aiiA* trangenic plants but not in control lines (Fig. 2b). Soluble protein samples extracted from transgenic tobacco leaves and potato tubers inactivated OHHL activity *in vitro* (Fig. 3a). Arbitrary enzyme activities of 7 tobacco and 5 potato lines ranged from 1 to 6 picomoles of OHHL hydrolysed by soluble protein
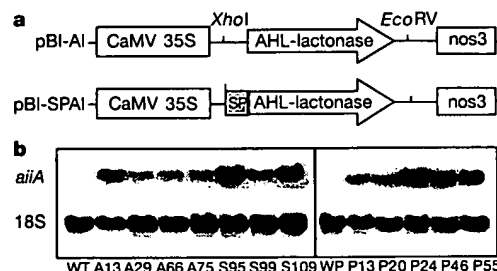


**Figure 1** ESI-MS and tandem mass spectrometry analysis of the hydrolysis product of OHHL. **a**, Electrospray ionization spectra of the AHL-lactonase hydrolysis product of OHHL (left column) and of the produced N-(3-oxohexanoyl)-L-homoserine (right column). We labelled the comparable peaks with their respective $m/z$. **b**, Mechanism of AHL-lactonase in inactivating OHHL. AHL-lactonase opened the homoserine lactone ring of OHHL in the presence of water to produce N-(3-oxohexanoyl)-L-homoserine.



**Figure 2** Genetic constructs for plant transformation and expression of *aiiA* messenger RNA in transgenic plants. **a**, Expression cassettes containing *aiiA* gene (pBI-AI) and the *sp–aiiA* fusion gene (pBI-SPAI). SP, signal peptide from the tobacco calreticulin; CMV35S, cauliflower mosaic virus 35S promoter; nos3, nopaline synthase transcription terminator. **b**, RNA gel blot analyses of *aiiA* in transgenic tobacco (left) and potato (right). We used a tobacco 18S rRNA (18S) probe to show relative RNA loadings. The A- and S-lines are tobacco *aiiA* and *sp–aiiA* transformants respectively; P-lines are potato *aiiA* transformants. WT, wild-type tobacco; WP, wild-type potato.

$(h^{-1} \mu g^{-1})$. The AHL-lactonase activities in plants transformed with the sp–aiiA fusion gene were generally lower than in those transformed with the aiiA gene.
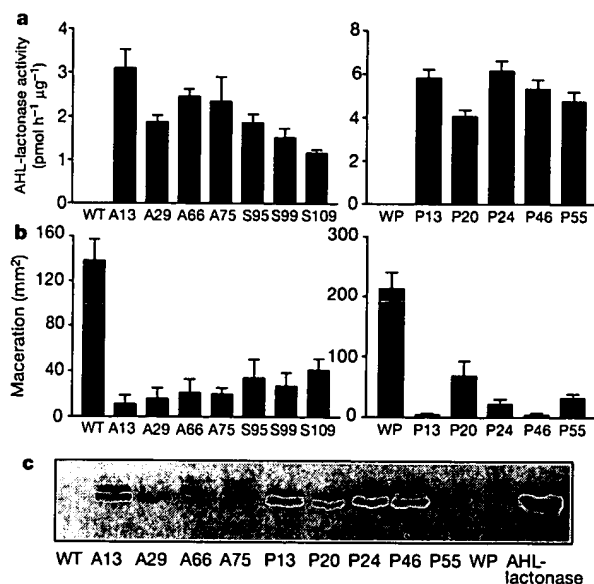
Figure 3b shows the areas of maceration of control plants and transgenic lines of tobacco and potato after inoculation with E. carotovora. Enhanced resistance of the aiiA/sp–aiiA transgenic plants is consistent with the ability of AHL-lactonase to inactivate OHHL (Fig. 3a). In transgenic lines with high levels of AHL-lactonase protein (Fig. 3c), for example, tobacco line A13 and potato lines P13, P24 and P46, the areas of maceration are less than 5% of those in untransformed controls (Fig. 3b), indicating a strong correlation between disease resistance and AHL-lactonase expression level. Transcriptional expression levels in the three sp–aiiA tobacco lines were higher than in the aiiA transgenic lines (Fig. 2b), but their OHHL-hydrolysing enzyme activities and resistance levels were not as strong as in the best aiiA transgenic lines (Fig. 3a, b). The enzyme activity of the purified SP-AHL-lactonase from Escherichia coli was comparable to that of AHL-lactonase. It is possible that the reduced levels of delactonase activity in the sp–aiiA trangenic plants resulted from trapping the SP-AHL-lactonase in microsomes, as in the case of calreticulin protein[23], which could slow down the speed of contact between the AHL-lactonase and OHHL.

Immunoblot analysis confirmed the translational expression of the AHL-lactonase protein (Fig. 3c). Lines with higher AHL-lactonase enzyme activities (for example, A13, P13, P24 and P46) also showed stronger immunoblot signals than the lines with the lower enzyme activities. By using dilutions of purified AHL-lactonase as standards in immunoblot analysis, we estimated the AHL-lactonase protein contents to be 2–7 ng mg$^{-1}$ and 20–110 ng mg$^{-1}$ of soluble proteins in tobacco leaves and in potato tubers, respectively.
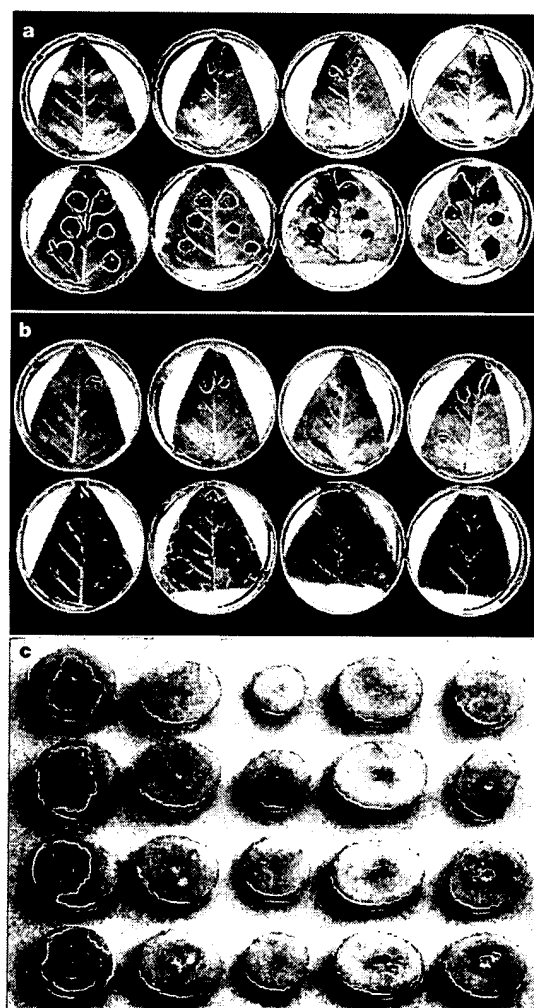
We found that resistance of the aiiA transgenic plants is related to the population density of the inoculated pathogen. Control plants including untransformed and GFP transgenic tobacco displayed typical maceration symptoms several hours after inoculation. The higher the population density of the inoculum, the larger the

maceration area on the tobacco leaves (Fig. 4a). In the four tested aiiA transgenic lines, a low density of inoculum (600 colony-forming units (CFU)) did not cause any symptoms 20 h after inoculation. Maceration occurred with these plants when they were inoculated with a high population density of pathogen (60,000 CFU), but the macerated areas were much smaller than in the controls. We obtained similar results with normal and transgenic potato lines when challenged with the pathogen at varied cell densities (25,000–100,000 CFU) (Fig. 4c).

Even when maceration was initiated in the aiiA transgenic lines after inoculation with a high population density inoculum, further symptom development was significantly retarded or even stopped. Forty hours after inoculation, the leaves of control tobacco were totally macerated, whereas symptoms on the aiiA tobacco leaves were almost the same as at 20 h after inoculation (Fig. 4a, b). The effect of AHL-lactonase on symptom development appears even



**Figure 4** Plant inoculation with Erwinia carotovora SCG1. **a,** Top row are tobacco leaves from aiiA lines A13, A29, A66 and A75; bottom row are those from untransformed lines W6, W7, W9 and a GFP-line G7, respectively (from left to right). We inoculated each leaf on 6 sites following the same order. The inoculum cell numbers on the two top spots, the two spots on the lower left side, and the two spots on the lower right side of each leaf were 60,000, 6,000 and 600 CFU, respectively. We took the photograph 20 h after inoculation. **b,** Same as a except that we took the photograph 40 h after inoculation. **c,** The potato tuber slices in columns from left to right are from wild-type plants and from aiiA lines P46, P24, P55 and P13, respectively. The inoculum cell numbers for rows from top to bottom were 25,000, 50,000, 75,000 and 100,000 CFU, respectively. We took the photograph 48 h after inoculation.



**Figure 3** AHL-lactonase enzyme activity and E. carotovora inoculation. **a,** AHL-lactonase enzyme activity in transgenic tobacco (left) and potato (right). The line designation is the same as in Fig. 2. The data are means of 5 (tobacco) or 8 (potato) replicates. Vertical bar represents standard error. **b,** Erwinia carotovora SCG1 inoculation assay. The population density per inoculation site was $6 \times 10^3$ CFU for tobacco or $1 \times 10^5$ CFU for potato. The data are means of 8 (for tobacco) or 6 (for potato) replicates. **c,** Immunoblot analysis of AHL-lactonase protein in transgenic plants.

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clearer in the *aiiA* transgenic potato. We observed watery rotten lesions in the control several hours after inoculation. Symptoms worsened progressively until the whole slice was completely macerated. In contrast, inoculation at high population density initially produced watery lesions in the tubers of *aiiA* potato but soon the lesions became dry and symptom development stopped (Fig. 4c). The probable pattern is that the AHL-lactonase by slowing down the pathogen from producing virulence factors, which include pectolytic enzymes, allows the host defense mechanisms to take over and eventually stop infection.

Our results demonstrate that enzymatic quenching of AHL quorum-sensing signals is a feasible approach for prevention of bacterial infection. Besides the two AHL-inactivation enzymes[11,17], two groups of AHL antagonists have also been documented. The halogenated furanones from marine red alga *Delisea pulchra* inhibited luminescence and virulence in *Vibrio harveyi* by displacement of OHHL from LuxR transcription factor[24-26]. The antimicrobial triclosan suppressed AHL biosynthesis through inhibiting the reaction catalysed by enoyl-acyl carrier protein reductase[27]. As the quorum-sensing regulation of virulence seems to be one of the common strategies that many bacterial pathogens have adopted during evolution to ensure their survival in host–pathogen interactions, this kind of quorum-quenching strategy could have wide applications in our fight against bacterial plagues. □

## Methods

### AHL-lactonase purification and structural determination

We purified the recombinant AHL-lactonase by using the glutathione *S*-transferase (GST) Gene Fusion System (Pharmacia) as described[11]. Briefly, the GST/AHL-lactonase fusion protein from *E. coli* cell-free extracts was bonded to glutathione affinity resins, and then recombinant AHL-lactonase was released from the bound GST protein by thrombin, a site-specific protease. Purity was determined by SDS–PAGE analysis. We mixed the purified AHL-lactonase (30 nmol) with OHHL (500 nmol) per milliliter of phosphate buffer (1/15 M, pH 8.0), and incubated the mixture at 28 °C for 10 min with gentle shaking. After incubation, we extracted the mixture three times with ethyl acetate, and evaporated the combined organic phase through a rotary evaporator. For HPLC analysis and purification, a sample was dissolved in 0.2 ml methanol and analysed with a symmetry $C_{18}$ reverse-phase column (4.6 × 250 mm). Fractions were separated by eluting isocratically with 50:50 methanol/water (v/v) at a flow rate of 1 ml min$^{-1}$. ESI-MS and tandem mass spectrometry were performed on a Finnigan/MAT LCQ ion-trap mass spectrometer. The sample dissolved in 50:50 methanol/water (v/v) was introduced into the mass spectrometer by loop injection. We synthesized all the AHLs used in this study as previously described[20]. We produced *N*-(3-oxohexanoyl)-L-homoserine by incubating the corresponding acyl-homoserine lactone (50 mg) in 350 µl dimethyl sulphoxide containing 350 µl of 1 M NaOH for 12 h at 25 °C. After incubation, we adjusted the mixture to pH 6.5 with 1 M NaH$_2$PO$_4$ and then extracted it three times with chloroform. The chloroform fractions were combined and evaporated to dryness. The product was purified by HPLC using a $C_{18}$ reverse-phase column. Its structure was confirmed by $^1$H NMR and mass spectrometry.

### Plant transformation and analysis

We cloned *aiiA* in a plant expression vector pBI121 that contains a kanamycin resistance gene as the selection marker and resulted in the construct pBI-AI (Fig. 2a; top panel). To test whether targeting of the AHL-lactonase enzyme into intercellular spaces could confer better resistance to *E. carotovora* infection, we prepared the second construct pBI-SPAI (Fig. 2a, bottom panel) by fusing the coding sequence of a 27-amino-acid calreticulin secretion signal peptide[23,28] to the 5' end of the *aiiA* coding sequence in the same open reading frame. We also cloned the coding region of the GFP in pBI121 to produce the construct pBI-GFP as a control. These constructs are under the control of the cauliflower mosaic virus (CMV) 35S promoter. We introduced the pBI-AI, pBI-SPAI and pBI-GFP constructs into the genome of tobacco (*Nicotiana tabacum* L. var. GX3) and potato (*Solanum tuberosum* L. var. Bintje) plants respectively through *Agrobacterium*-mediated transformation[16,17]. Transgenic plants were grown under standard conditions (25 °C, 16/8 h light/dark cycle) in growth chambers.

We extracted total RNA from tobacco and potato leaves by the Trizol method (Gibco). Total RNA (10 µg) was fractionated in 1.2% formaldehyde agrose gel and transferred to Hybond-N$^+$ (Amersham) nylon membranes. The *aiiA* coding region and the 18S ribosomal RNA fragment of tobacco were labelled separately with α-P$^{32}$-deoxycytidine 5'-triphosphate (α-P$^{32}$-dCTP) using Rediprime II (Amersham). Hybridization was performed according to standard procedures.

We determined AHL-lactonase enzyme activity by incubation of OHHL with total soluble protein samples extracted from plants. Fresh tobacco leaf tissue or potato tuber tissue (1 g) was homogenized with 1.5 ml (for tobacco) or 1.0 ml (for potato) of protein extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$, 18% (w/v) sucrose, 40 mM 2-mercaptoethanol). Reaction mixture containing protein extracts and OHHL (40 µM)

was incubated for 30 min at 28 °C. After digestion, we determined the remaining OHHL as previously described[11] and calculated the digested OHHL. AHL-lactonase activity is defined as picomoles of OHHL hydrolysed per hour per microgram of total soluble protein.

We prepared polyclonal anti-AHL-lactonase rabbit antiserum by multiple injections of the purified AHL-lactonase protein into rabbits. For immunoblot analysis, we separated soluble proteins from wild-type and transgenic plants as well as the purified AHL-lactonase protein (10 ng) by SDS–PAGE and visualized with anti-AHL-lactonase antibodies and a standard alkaline phosphatase immunoassay.

### Plant inoculation

We prepared *E. carotovora* pv. *carotovora* SCG1 cells by centrifugation (5,000g for 10 min) of overnight culture and dilution to different population densities in phosphate buffer (1/15 M, pH 8.0). We excised tobacco leaves of the same age from 5–8-week-old plants and placed them in Petri dishes that contained one layer of Whatman filter paper moistened with 2 ml of sterile water. We inoculated the leaves by adding 3 µl SCG1 dilution on the freshly wounded leaf surface (punched slightly with a 20-µl pipette tip). For potato, we surface-sterilized mature tubers with 70% ethanol, sliced them 3 mm apart, and placed them on Petri dishes with wet filter paper to keep them moist. To each slice we added 5 µl SCG1 dilution after gentle tip punching, and incubated them at 28 °C for 20 h (tobacco) or 48 h (potato), unless otherwise indicated, before determining the extent of maceration.

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# Production of multiple plant hormones from a single polyprotein precursor

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Some animal and yeast hormone genes produce prohormone polypeptides that are proteolytically processed to produce multiple copies of hormones with the same or different functions[1]. In plants, four polypeptides have been identified that can be classed as hormones[2-5] (intercellular chemical messengers[6]) but none are known to be produced as multiple copies from a single precursor. Here we describe a polyprotein hormone precursor, present in tobacco plants, that gives rise to two polypeptide hormones, as often found in animals and yeast. The tobacco polypeptides activate the synthesis of defensive proteinase-inhibitor proteins in a manner similar to that of systemin, an 18-amino-acid polypeptide found in tomato plants[2]. The two tobacco polypeptides are derived from each end of a 165-amino-acid precursor that bears no homology to tomato prosystemin. The data show that structurally diverse polypeptide hormones in different plant species can serve similar signalling roles, a condition not found in animals or yeast.

Following the discovery of systemin in tomato leaves, homologues were identified in potato, nightshade and pepper[7], but not in tobacco. Tobacco exhibits a systemic wound induction of proteinase inhibitors[8] similar to that found in tomato, potato and pepper, but we could not detect a systemin-like polypeptide or its precursor in tobacco leaves. (The term 'systemin' is used to describe polypeptide defence signals that are produced by the plant in response to physical damage and that activate defence genes, either locally or systemically.) Systemic responses to herbivore attacks have been documented in more than 100 species of plants[9], and the chemical nature of these signals is pertinent to the evolution of information-processing systems in plants as well as agricultural, biotechnological, environmental and ecological applications. We therefore initiated a search for the tobacco wound signal, hoping that the isolation of a tobacco systemin would provide a clue to the diversity of systemic wound signals within the Solanaceae, and would lead to the identification of polypeptide defence signals in other plant families.

We developed an assay to identify and isolate the systemin-like polypeptide of tobacco with suspension-cultured tobacco cells, on the basis of the ability of tomato systemin to cause an increase in the pH (alkalinization) of the culture medium[10,11]. Aliquots of cultured cells (1 ml) were used to assay alkalinization activity in either crude extracts or in individual fractions from chromatographic columns during purification (see Supplementary Information 1). Aliquots of 1–10 µl from the fractions were added to the agitated cells and the pH was monitored. The pH of the suspension cell medium increases in response to increasing quantities of a crude tobacco leaf extract (see Supplementary Information 2). Increases in the medium pH in response to aliquots of fractions from high-pressure liquid chromatography (HPLC) (see Supplementary Information 1, Step 4) clearly identified two major peaks and one minor peak that eluted from the column (Fig. 1a). Each major peak was further analysed by HPLC, producing purified polypeptides I and II (Fig. 1b; see Supplementary Information 1, Step 6).

The biological activities of polypeptides I and II were assayed in tobacco suspension cultures and in leaves of tobacco plants. The alkalinization of the tobacco cell cultures in response to both polypeptides peaked at about 15 min (Fig. 2a) and then slowly declined. Tobacco polypeptides I and II exhibited similar concentration dependence for the induction of alkalinization of cell culture medium (Fig. 2b) and for the induction of tobacco trypsin inhibitors in leaves (Fig. 2c). Tobacco trypsin inhibitors are members of the potato inhibitor II family[8,12]. Members of this family are induced by wounds, methyl jasmonate and systemin in tomato[13]. Tomato systemin did not cause an alkalinization of the tobacco cell-culture medium, nor did it activate the synthesis of tobacco trypsin inhibitor in leaves when supplied to young tobacco plants through their cut stems, confirming that tomato systemin is not perceived by tobacco cells. Whether tobacco systemins are local signals, systemic signals or both has not yet been established. In tomato plants, an antisense prosystemin severely reduced systemic signalling[14]. Transformation of tobacco plants with an antisense precursor gene for tobacco systemin should be helpful in this regard.

Polypeptides I and II contained 18 amino acids each and were
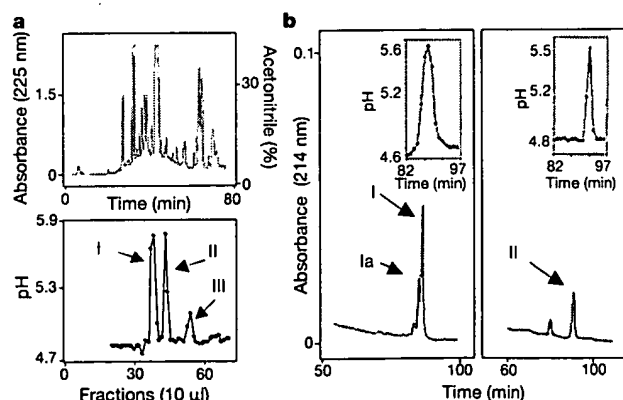


**Figure 1** Isolation of biologically active tobacco polypeptides from tobacco leaf extracts. **a**, Reversed phase C18 HPLC separations of partially purified tobacco leaf extracts (top panel) and the assay of 10 µl of each eluted fraction (2 ml) for their ability to cause the alkalinization of 1 ml of the medium of tobacco suspension cultured cells (lower panel). The pH of the culture medium was recorded after 15 min. **b**, Final purification of polypeptides in peak I (left) and peak II (right) from **a** by narrow-bore reversed-phase chromatography. From each 0.25-ml fraction, 1 µl was assayed for its alkalinization activity (insets).

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